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ANNUAL REPORT

STRESSOR CONTROLLABILITY AND IMMUNE FUNCTION
ONR GRANT No. N0014-85-K-0411

I. Scientific Goals

Recent evidence has increasingly focused on complex psychological factors such as the ability to "cope" as critical factors in determining the behavioral and physiological impact of exposure to stressors. This may also be true for the immunological consequences of stressor exposure. The "learned helplessness" paradigm provides a potential animal model in which this process can be studied. Here the organism's ability to exert behavioral control (a form of coping) over an aversive event is manipulated, and the impact of control or the lack of it can be separated from the impact of exposure to the physical stressor itself.

The objectives of the proposed research are to establish a reliable paradigm for producing immune system alteration by a stressor, to determine the role of behavioral control/lack of control, and to study the processes involved. There are three specific aims:

1. To explore the nature of the critical behavioral factors which lead to immune alteration.
2. To determine the physiological changes produced that are responsible for the immune changes.
3. To study the nature of the immune change itself.

II. General Scientific Goals for the Year September, 1985 to July,1986

Two major changes in direction are planned. One change concerns the behavioral procedures used to produce immune system changes. We plan to switch from a focus on inescapable shock to defeat in aggressive encounters. The second change concerns immunological procedures. We plan to switch from a focus on in vitro to in vivo measurement. The details of these changes and the reasons for them will be easier to describe after describing progress to date. I will therefore return to a discussion of these changes later.

III. Summary of Research for July, 1984 to September, 1985

1. Learned Helplessness.

In an initial series of studies we attempted to determine whether stressor controllability might be an important factor in modulating the impact of exposure to a stressor on immune function. Rats were given a single session of escapable, yoked inescapable, or no electric shocks. The rats were placed in small boxes with a wheel located on the front wall which the rat could turn. The rat's tail extended out of the box and had shock electrodes attached. Escape rats were exposed to a series of 80 1.0 mA shocks each of which could be terminated by turning the wheel a complete rotation. Thus the Escape rats had control over the

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The purpose of this project is to isolate psychological factors that modulate the impact of stressors on immune function and to study the mechanisms involved. The research focuses on developing a paradigm in which coping factors can be manipulated and their impact studied. Theory and data suggest that behavioral control over a stressor is a major coping process. Here control over shock is manipulated by exposing rats to escapable (controllable) or exactly equal amounts and distributions of inescapable (uncontrollable) shocks. Inescapable shocks reduced lymphocyte proliferation to PHA and ConA, and suppressed natural killer cell cytotoxicity. Escapable shock had no effect at all, even though physically identical in intensity, duration, and distribution. Thus control/lack of control rather than shock per se was critical. This effect was not mediated by pituitary-adrenal hormones. Current work focuses on 3 aspects: 1) The development of a more ecologically valid stress paradigm using defeat; 2) The assessment of in vivo immune functioning, and 3) endogenous opiates as potential mediators.

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termination of shock--their behavior determined the duration of each of the shocks. A second set of rats was yoked to the first and received inescapable shocks. Each member of this group was paired with a member of the Escape group. Each shock began for the Inescapable subject at the same moment as for the Escape animal, but wheel turning here had no effect on the termination of shock. Shock terminated for the Inescapable animal whenever the Escape animal responded. Thus the Escape and Inescapable animals received the identical durations and distributions of shock, but the Escape subject had an element of behavioral control (its behavior determined shock durations), while the Inescapable animal had no control. The initial experiments also had separate restrained and home cage controls.

Twenty-four hr later the animals were given a very brief reexposure to shock and blood samples and spleens were taken. This reexposure procedure was used because prior work had indicated a maximal response with this procedure using other measures. Mitogen induced lymphocyte proliferation and natural killer cell cytotoxicity were examined. Proliferation to the T cell specific mitogens PHA and ConA were influenced by shock, but only in the animals that did not have control over the shock. Proliferation was suppressed in the animals that had received inescapable shock but was completely unaffected in the animals that had received escapable shock. Similarly, natural killer cell cytotoxicity was reduced by inescapable but not by escapable shock. Thus the controllability/uncontrollability of the stressor rather than mere physical exposure to the stressor seemed to be the critical variable in determining whether the shock stressor altered immune function. The Escapable and Inescapable animals received identical exposures to shock, yet only the Inescapable animals showed changes on the measures of immune function taken.

2. Pituitary-adrenal Activity.

An obvious hypothesis to explain our basic findings is that uncontrollable shock might produce higher adrenal corticosteroid levels than does controllable shock, thereby resulting in immunosuppression. We thus have used standard RIA techniques to monitor both serum corticosterone and ACTH following controllable and uncontrollable shock. The full timecourse following shock of both hormones was observed. Neither peak level nor rate of dissipation differed following controllable and uncontrollable shock (Figures 1 and 2). It was possible that even though uncontrollable and controllable shock did not produce initial differences, the pituitary-adrenal system might have been differentially sensitized. Thus we gave animals uncontrollable or controllable shock, reexposed them to a small amount of shock 24 hr later, and tracked the timecourse of corticosterone and ACTH. Again, there were no differences (Figures 3 and 4) between animals which had received controllable and uncontrollable shock. It is thus extremely unlikely that the differences in lymphocyte proliferation and NK activity produced by uncontrollable and controllable shock could be mediated by adrenal corticosteroids.

3. T Subset Determination.

We have now developed the procedures for labeling rat lymphocytes with monoclonal antibodies directed against cell surface markers. A lymphocyte suspension, containing at least 1 X 10^5 cells/ml,

is divided into 0.2 ml aliquots after washing and separation on Ficoll Paque. The suspension is added to each of five 1.5 ml microcentrifuge tubes. To tubes 1-4, monoclonal antibodies, obtained from Donald Bellgrau, are added. The antiodies which we have not tested include W3/13 (pan T-cell), OX6 (B-cell), W3/25 (helper), and OX8 (suppressor). The antibodies obtained from cell line supernatants are added undiluted in volumes of 0.1 ml (W3/13 and OX6) or 0.5 ml (W3/25 and OX8), and the suspension is incubated for 45 min in a continuously shaken ice bath. After incubation the cells are washed three times in cold Hank's balanced salt solution (HBSS) and resuspended to .45 ml in cold HBSS. The second antibody (fluorescein-conjugated goat antimouse IgG with no cross-reactivity with rat) is then added in a 0.9 ml volume diluted 1:1000. The fifth tube receives only the second antibody for the determination of nonspecific binding. Tubes are then incubated for 45 min in a continuously shaken ice bath. After incubation the cells are washed three times in cold HBSS. A sixth tube containing unlabeled cells is also preserved in paraformaldehyde for determination of sizing parameters on the flow cytometer. Initial studies have indicated that cells fixed in this manner can be stored for up to two months at -5° without a loss of the label.

Preliminary results obtained from a population of control rats is indicated in Table 1. The Coulter Epics C flow cytometer was gated on small lymphocytes. We are currently determining whether inescapable shock alters T subset ratios.

TABLE 1. Percentage of rat lymphocytes labeled by monoclonal antibodies obtained from Bellgrau.

W3/13	W3/25	OX8	OX6
76.5 +/- 7.0[1]	57.1 +/- 7.0	31.9 +/- 10.0	12.8 +/- 4.4

[1]Mean +/- S.D., N = 10

4. Antibody Development Following Immunization with KLH.

Many investigators have noted a variety of interpretive difficulties with *in vitro* measures of immune function. The most frequently noted problem is that standard measures such as mitogen induced proliferation do not provide a clear indication of how the system as a whole might function *in vivo*. THe fact that lymphocyte proliferation might be suppressed does not really mean that the immune system would be less responsive to an invading pathogen or less effective in dealing with it. For this and other reasons we undertook to develop an *in vivo* measure. Because a major end-point of the immune system is antibody production to an antigen, we decided to measure this aspect.

Keyhole limpet hemocyanin (KLH) is highly immunogenic in a variety of species, indlucing the rat. An enzyme linked immunosorbent

assay (ELISA) has been developed which permits the determination of plasma IgG antibody levels to KLH in animals following immunization.

a. ELISA Procedure.

Wells of a flat bottomed microtiter plate (NUNC, certified Immunoplate I) were coated with KLH (0.2 ml/well, 0.5 mg KLH/ml bicarbonate buffer) by incubating overnight at 5° C. The following day the plates were washed three times with Tween phosphate buffered saline (T-BPS), shaken out, sealed with plastic tape, and stored at -20° C until needed. Postblocking with a second protein has not been necessary since background absorbancy has been quite low.

The ELISA was performed as follows: plasma samples were serially diluted in the wells of the microtiter plate from 1:1000 to 1:128,000 in a final volume of 0.2 ml. The plate was sealed with plastic tape and incubated at 37° C for three hours. At the end of the incubation all wells were washed three times with T-PBS. Next, 0.2 ml of the enzyme-antibody conjugate (diluted 1:3000) was added to the wells, the wells were sealed again, and the plates were incubated at 37° C for one hour. The plates were washed again three times with T-PBS. Finally 0.2 ml of enzyme substrate [1 mg substrate (Sigma reagent 104)/ml buffer, pH 9.8] was added to the wells. Color was allowed to develop in the dark for 15 min: 0.1 ml of 1.5 M NaOH was added to all wells to stop the reaction. Forty-five minutes later the plate was read on an ELISA plate reader set at 405 nm.

b. Immunization Procedure.

Rats are immunized with 1.25 mg KLM suspended in sterile saline by subcutaneous injection in the scapular region. Heparinized blood samples are obtained from unanestnetized animals from the tail or by cardiac puncture from ether anesthetized animals. The method of sampling appears to have no effect on the antibody titers measured. Animals receive a primary immunization followed sixty days later by a secondary immunization. Preliminary results for non-germ free animals are given in Table 2. The changes in antibody levels, not surprisingly, represent statistically significant differences across time [$F(2,34) = 61.01$, $p < .01$]. Importantly we have noted no behavioral pathology following the immunization nor signs of general physical debilitation or anaphalytic shock.

TABLE 2. IgG antibody levels specific for KLH in plasma diluted 1:1000 as measured by an ELISA procedure. KLH antibody titers are reported as absorbence units at 405 nm.

Preimmunization	Two Weeks Postimmunization	Presecondary	Two Weeks Postsecondary
Not detectable	0.179+/-0.082[1]	0.460+/-0.09	0.584+/-0.078

[1]Mean +/- S.D.; N = 14

5. KLH Antibodies and Learned Helplessness.

Animals were assessed for baseline levels of KLH antibodies and then immunized with 1.25 mg sc in .25 cc of saline. They were then exposed to inescapable electric shock described above. KLH antibodies were measured one week and two weeks later. Antibody generation was unaffected by our shock treatments. These results are shown in Figure 5. Results from secondary immunization are not yet complete.

6. Difficulties Encountered.

A number of major difficulties have been encountered which have significantly slowed the progress of this research. The first is that the in vitro assays that we have used entail a great deal of variability, both within a group of subjects and from animal shipment to shipment. Rats taken from the same shipment can differ enormously in lymphocyte proliferation and NK cell activity. Two shipments can have completely different baselines. These facts make it very difficult to do repeatable studies across time.

We have taken several approaches to try and deal with this problem. An obvious possibilty was that the measures were so variable because the rats were being infected with viruses and other agents that could alter proliferation. We thus began to purchase pathogen-free animals and to house them in a colony separated from all of the others in the animal facility. A variety of procedures have been attempted. For example, we have tried to use filter-tops for the cages. It might be noted that the use of filter tops actually suppressed proliferation, possibly because of the restriction of air-flow (see Figure 6). Isolating the animals from the rest of the colony and requiring the staff to service this room at the beginning of the day before other colonies have been visited has proved somewhat succesful. Variability has been reduced, although it is still high.

However, even when the assays worked well and variability was reasonable, the effect of inescapable shock was weak and not always present. We have manipulated a number of variables such as number of shocks, shock intensity, and number of days of shock exposure (1, 2, or 3). We have not found a procedure which produces a satisfactorily consistent effect. It should be noted that we have not attempted to use extremely intense or prolonged shock exposures. This is because our aim is to study the imapct of the psychological dimension of the lack of control, not the outcome of exposure to very extreme conditions.

7. Changes in Direction.

These difficulties have led us to change direction in two ways. On the immunological level, we will concentrate on in vivo measures such as antibody production and will de-emphasize in vitro measures such as lymphocyte proliferation. Antibody production to a previously unencountered antigen is more clearly related to the functioning of the system and in our hands is much less variable.

On the behavioral level, we will move away from shock paradigms. We propose to study the effects of defeat in agressive encounters. Defeat is chosen for a number of reasons. 1) There is considerable evidence that defeat may induce the same changes as does inescapable shock. For example, it produces the same later learning deficits, the same opioid

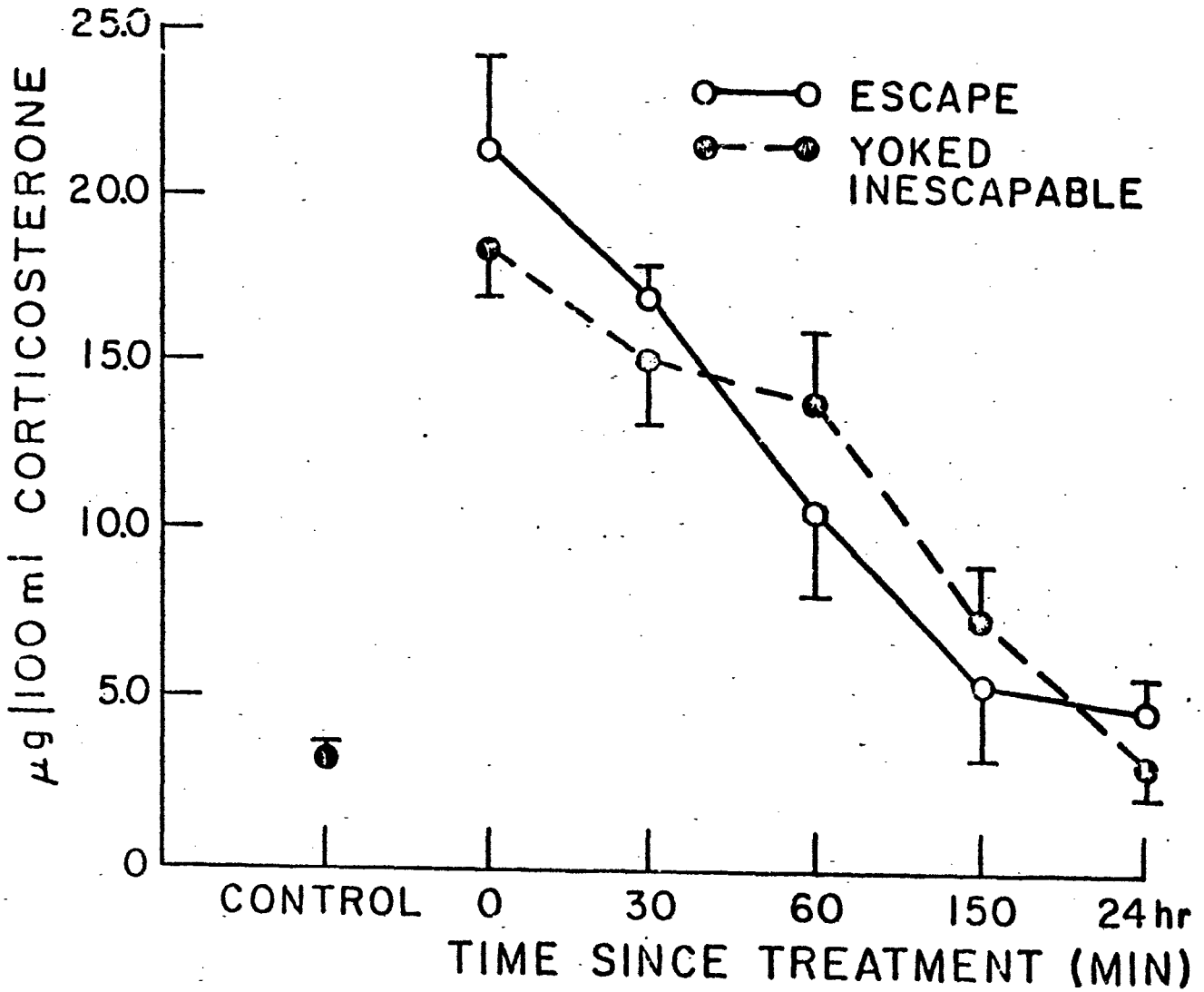
changes, etc. Moreover, inescapable shock and defeat summate in their effects. Thus prior exposure to inescapable but not escapable shock facilitates defeat in aggressive encounters and prior defeat exacerbates the reactions to inescapable shock. 2) Aggression is a form of coping, just as is control. Thus for example, animals allowed to aggress during exposure to a stressor such as shock show smaller stress responses (e.g., plasma corticosterone) than do animals not allowed to aggress. 3) Defeat is a "naturally" occurring event, in contrast to shock. 4) Defeat is easy to manipulate and may be easier to use than inescapable shock to produce immune changes. For example, repeated exposures may increase the likelihood of finding changes. However, multiple sessions of inescapable shock may not be very useful because the animals adapt to shock. They do not seem to adapt to defeat in the paradigm which we are currently using and so this procedure seems promising.

In our first attempt at producing defeat we confronted a lactating female with an unfamiliar male. A number of reports indicate that a lactating female will attack and defeat an unfamiliar male introduced into her home cage. We were unsuccessful in producing consistent aggression in this fashion. I then consulted a number of aggression experts and they recommended a different procedure--colony-intruder attack. In this paradigm two males and a female are allowed to live in the same environment (large tub cage) for a month. One of the males will become dominant during that period of time. An intruder is then introduced. The dominant male will attack the intruder with a fairly short latency. The intruder is kept in the cage for a short period (10 min) and is then removed. During this period attack will ordinarily have occurred and it is ordinarily enough to induce submissive posturing in the intruder. The intruder can be returned for any number of sessions. The advantage here is that after a small number of sessions (1 or 2) the intruder adopts a defeat posture as soon as he is introduced and actual attack does not occur. Thus any number of defeat sessions cam be employed <u>without physical injury or even contact</u>. An experiment manipulating number of sessions of defeat designed to determine whether defeat will produce immune alterations is now in progress. If succesful this paradigm will be used and efforts will concentrate on the role of endogenous opiates.

IV. Publications

Maier, S. F., Barksdale, C. M., & Kalin, N. H. (1986). Stressor controllability and the pituitary-adrenal system. <u>Behavioral Neurosciences</u>, in press.

Laudenslager, M. L. (1966). Psychosocial stress and susceptibility to infectious disease. In E. Kurstak, P. V. Morozov, & Z. P. Lipowski (Eds.), <u>Viruses, immunity, and mental health</u>. Plenum Press, in press.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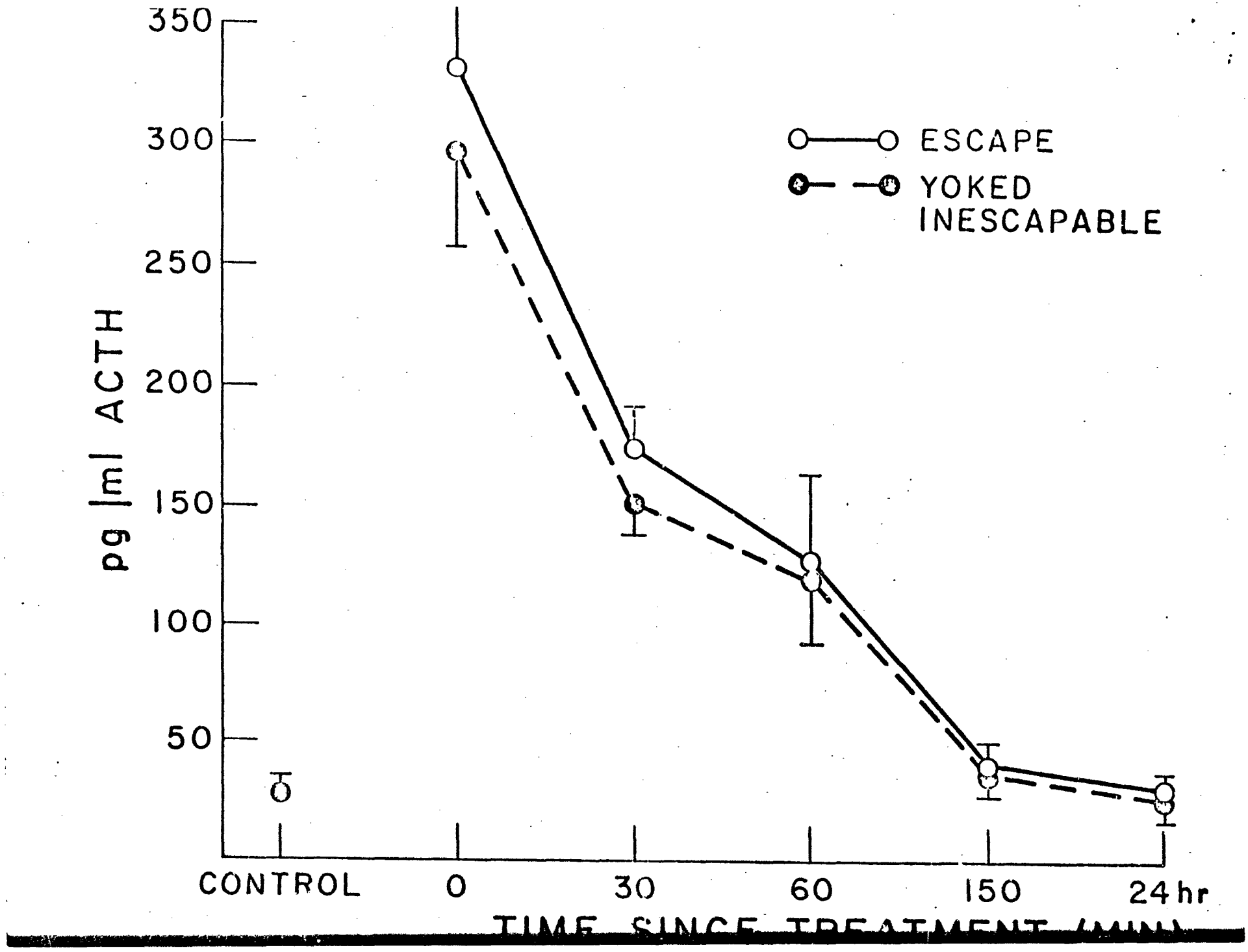
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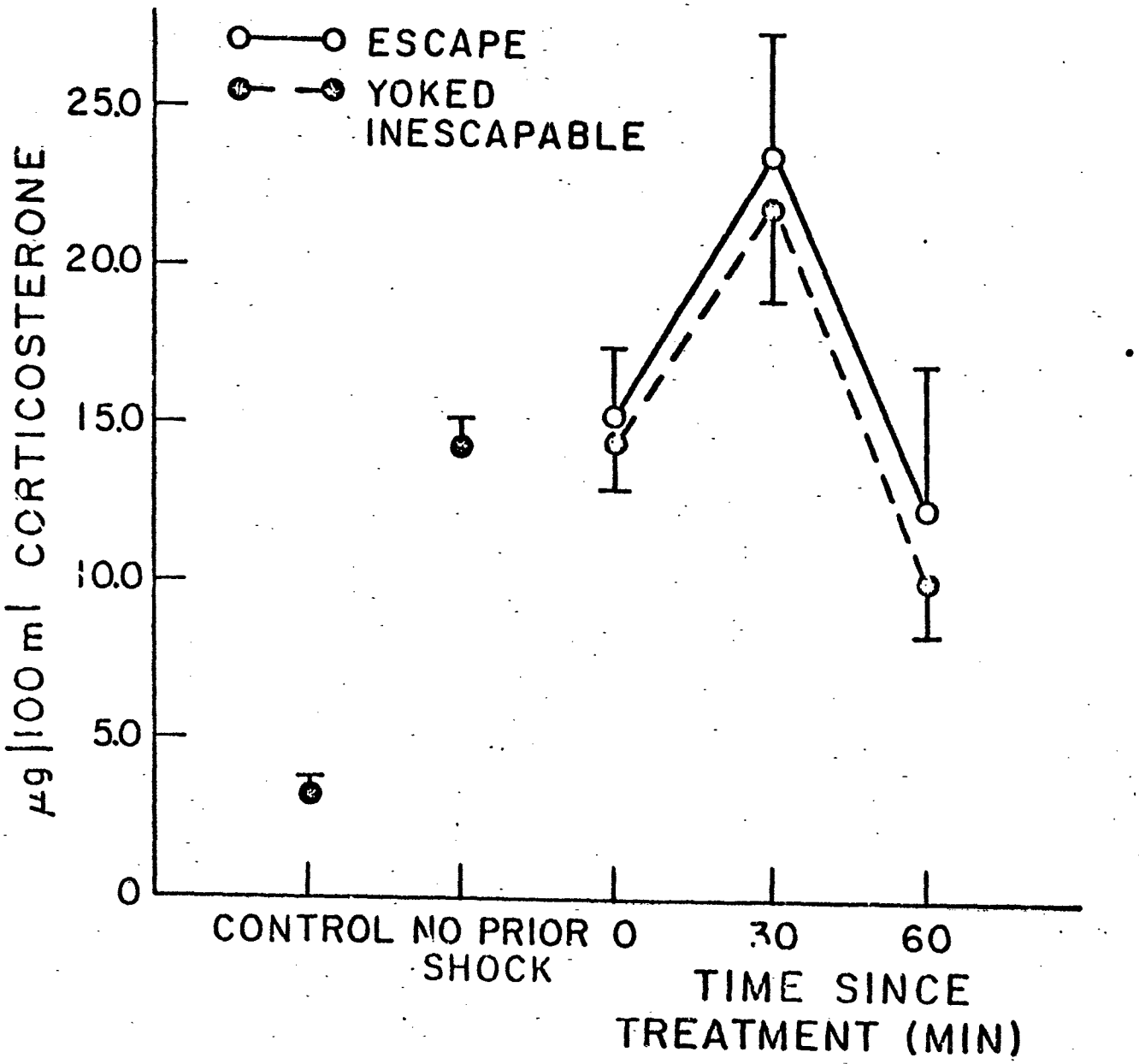
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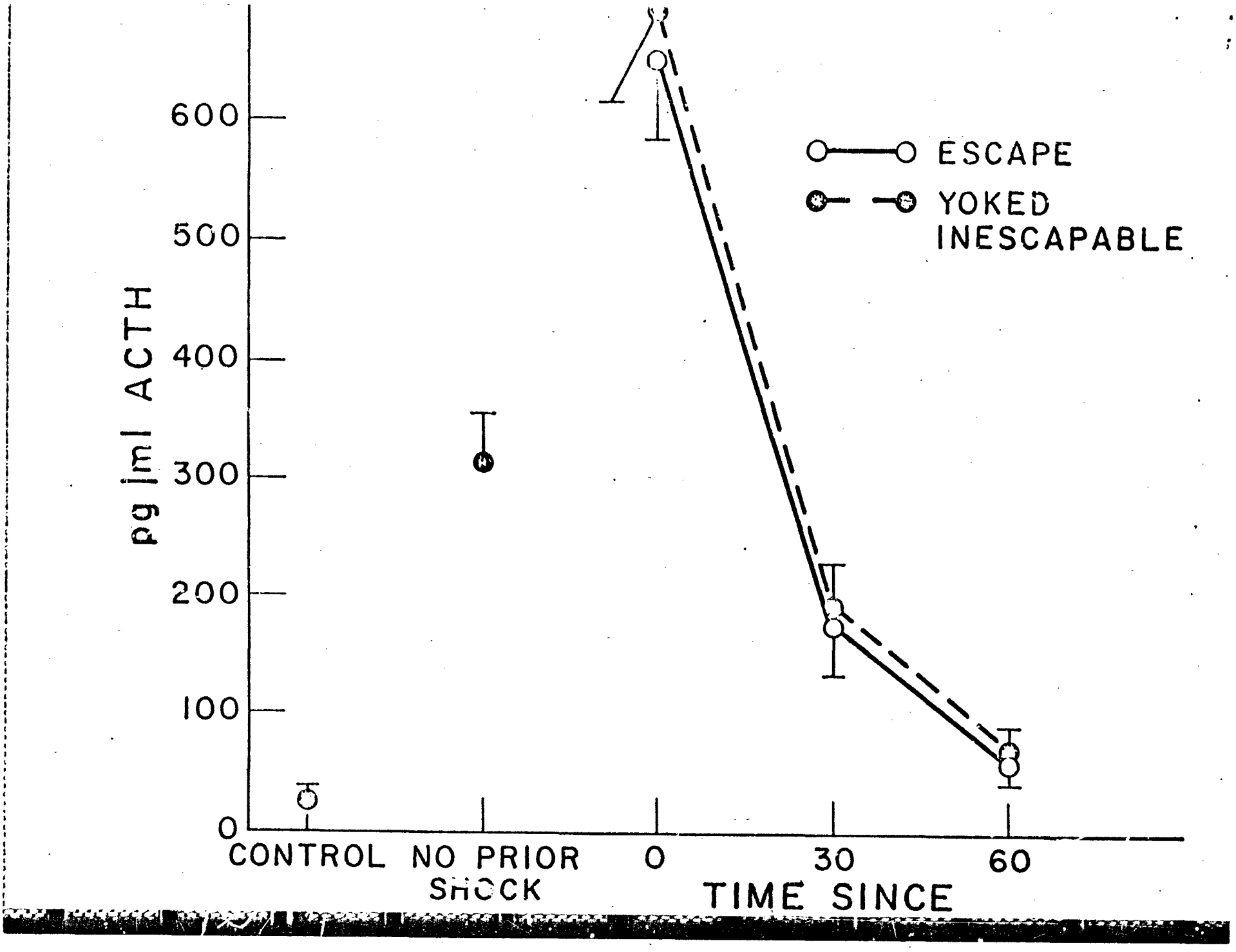
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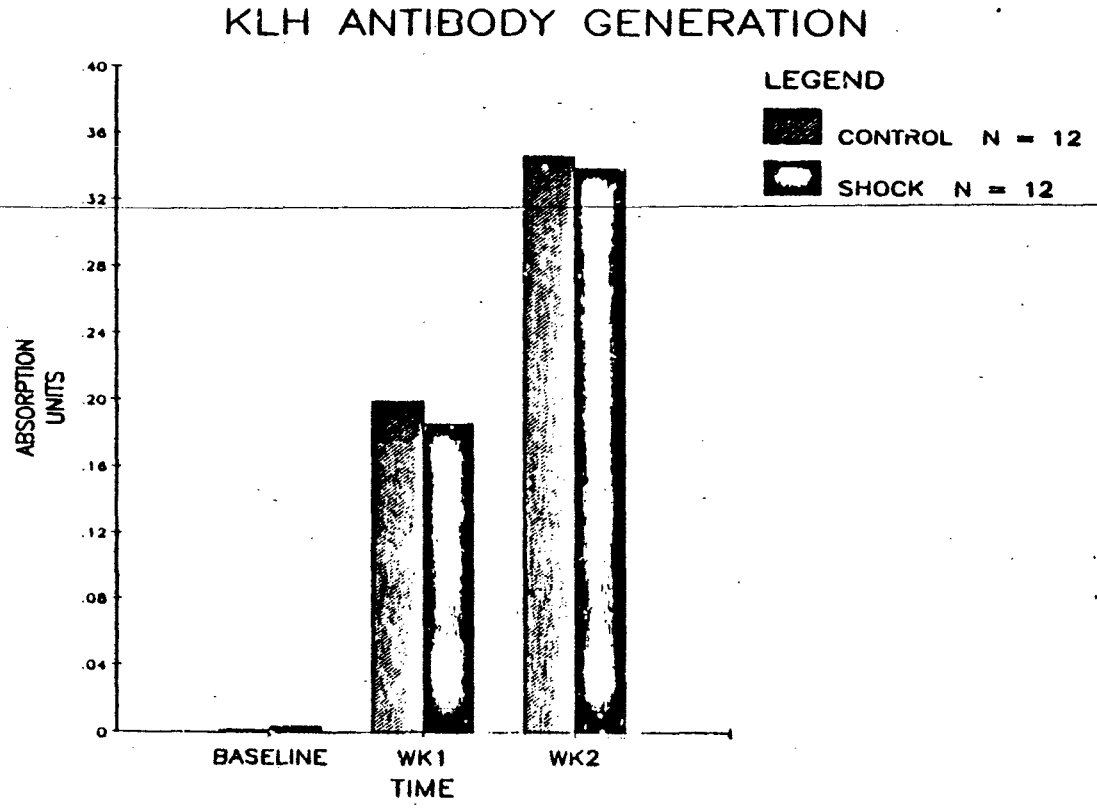
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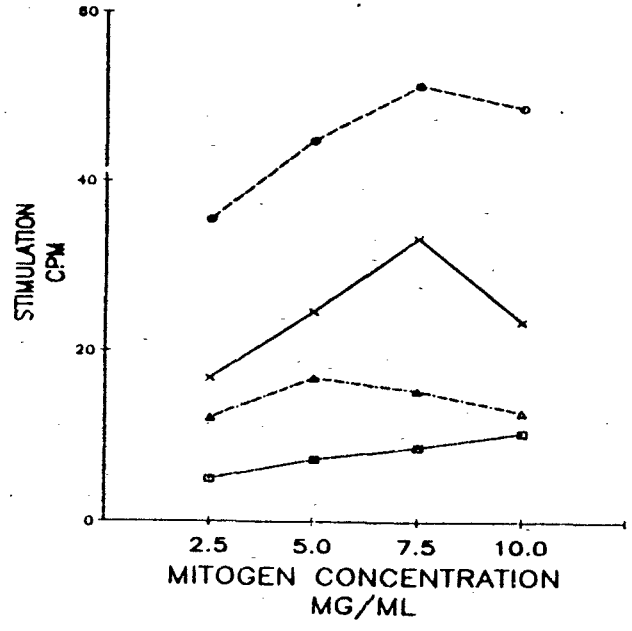

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